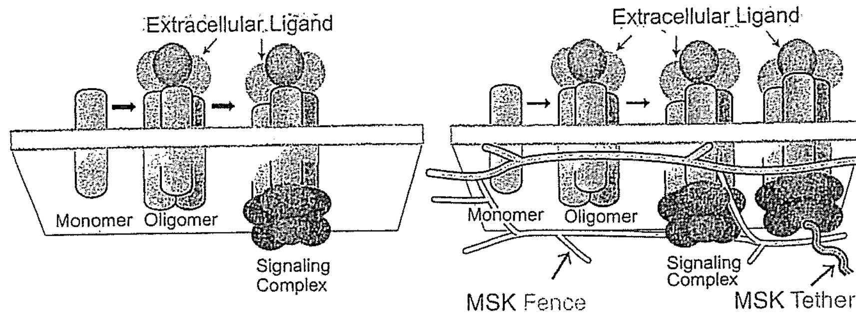

Fig. 13.3 Oligomerization-induced trapping model for membrane molecules upon oligomerization or molecular complex formation. Upon oligomerization or molecular complex formation, the hop rate across the intercompartmental barrier would be reduced greatly (right), because, in contrast to monomers, in the case of molecular complexes, all of the molecules within the complex have to hop across the picket-fence line simultaneously. In addition, due to the avidity effect, molecular complexes are more likely to be tethered to the membrane skeleton, perhaps temporarily, which also reduces their overall diffusion rate. The enhanced confinement and binding effects induced by oligomerization or molecular complex formation are collectively termed "oligomerization-induced trapping" (Iino et al. 2001). This would not occur in the absence of membrane skeleton fences and pickets (left): the diffusion theory by Saffman and Delbrück (1975), based on the fluid-mosaic model of Singer and Nicolson (1972), predicts that the diffusion rates of the oligomeric complexes would be almost the same as those of the single receptor molecules.

reorganization of the cytoskeleton. Categories 3 and 4 indicate the two major ways of scaffold formation for molecular interactions in and on the membrane: Category 3 emphasizes the lipid–lipid and protein–lipid interactions, whereas Category 4 mainly addresses the protein–protein interactions. The cells may take advantage of these two categories of domains, depending on the required number of molecules and molecular species, the levels of specificity in the molecular interaction and the lifetime of the complex. However, the boundary between these two types of domains is somewhat vague, because they tend to be mutually dependent on each other and one interaction tends to enhance the other.

effectively confine lipids, proteins, and virtually all of the molecules incorporated in the membrane. A Monte-Carlo simulation showed that when 20–30% of the compartment boundaries are occupied by anchored transmembrane protein pickets, temporary confinement of a lipid molecule on the order of 1–25 ms, as found in experiments, could be reproduced. This amount of bound transmembrane protein is consistent with the overall binding of 15% of the transmembrane proteins to the membrane skeleton. Since the membrane viscosity is high, about 100-fold greater than that in water, the presence of an immobile picket reduces the diffusion rate around it by several nanometers and thus the compartment boundaries do not have to be completely closed by the pickets. The average gap between immobilized anchored pickets is thought to be 3–10 nm or for the passage of the raft domain through the gap the effective size of the gap may be 2–9 nm, due to the exclusion of cholesterol from the boundary domain around the transmembrane proteins.

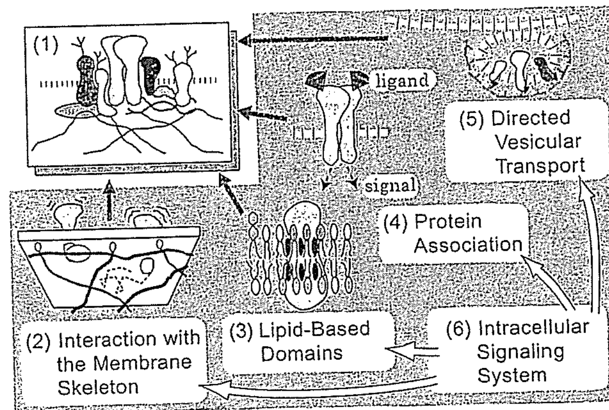

Fig. 13.4 Molecular processes that form the basis for the dynamics, structure and function of membrane molecules in the plasma membrane. (1) Multimolecular interactions forming large, stable membrane domains of Category 1. (2) Interaction with the membrane skeleton and associated transmembrane protein pickets, inducing membrane partitioning and the compartments of Category 2. (3) Lipid-based molecular interactions that may lead to the formation of raft domains (Category 3). (4) Protein oligomerization and complex formation, for example, induced by ligand binding (Category 4). This is likely to be a critical trigger for the initiation of a variety of membrane processes. This is also a key elementary process that greatly enhances the other molecular interactions described in this figure. (5) Directed vesicular transport, which may play crucial roles in the creation of concentration variations and specific membrane domains in the plasma membrane. (6) Intracellular signaling systems, which may be necessary for coordinating these molecular processes. This figure also shows that large, stable membrane domains may be generated by the actions of various elementary processes depicted here.

We further stress that these four classes of domains are interactive. Take the case of the creation of large, stable domains (Category 1) as an example (see Fig. 13.4). Molecular complex formation (Category 4) may lead to the stabilization of lipid raft domains (Category 3), and to confinement within the existing membrane skeleton compartment and/or transport to specific places by the membrane skeleton (Category 2). Such assemblies of molecules in the plasma membrane might act as platforms to recruit cytoplasmic molecules, perhaps including molecules loaded on the transport vesicles (shown as "(5)" in Fig. 13.4). In addition, signaling mechanisms may coordinate the various recruitment processes (shown as "(6)" in Fig. 13.4).

13.4 The Cell Membrane is a Two-dimensional Non-ideal Liquid Containing Dynamic Structures on Various Time-Space Scales

In the previous sections, we emphasized the presence of various membrane domains in the plasma membrane. However, at the same time, particularly in the context of raft domains and protein oligomers (molecular complexes), it is im-

portant to realize that the plasma membrane is not a simple liquid, but rather a non-ideal liquid mixture of molecules with various levels of miscibilities (in addition, it contains immobile molecules and domains that may be bound to the underlying membrane skeleton). Thus the plasma membrane naturally contains dynamic structures (like molecular complexes and domains), existing in various time and space scales, that are forming and dispersing continually within the cell membrane. As described in Section 13.1, these molecular complexes and domains range from protein clusters of small sizes with short lifetimes, like transient dimers of rhodopsin (Kusumi and Hyde 1982), to large micron-sized stable domains, like desmosomes (Pasdar and Nelson 1988a,b, 1989). Perhaps, the entire membrane should be viewed as a mosaic of microdomains (Maxfield 2002; Pierini and Maxfield 2001).

Based on the concept of a non-ideal liquid, we argue that the plasma membrane is always prepared for the formation of various domains and molecular clusters with enhanced sizes and lifetimes. We think that this concept is really a key toward understanding how the raft may be involved in the signaling and trafficking of raftophilic molecules.

13.5
A Definition of Raft Domains

Among the various membrane (micro)domains, the most controversial one is the so-called raft domain (Mukherjee and Maxfield 2004). From here, we now concentrate on raft domains. The first problem of writing about raft domains is that it is a word that has not been defined well. Lai called it an "unidentified floating object" (Lai 2003). Many researchers in related fields are uncomfortable with the status and the way the raft research field is being developed, and hastily demand a definition of the raft before one studies or talks about it. However, this seems to be a time to be patient about the *status quo* of raft research. The term "raft domain" is like the term "biophysics", which vaguely defines a research area: "biophysicists" basically know what it is (but with many grey areas), although they cannot specifically define it, and researchers in adjacent areas are often skeptical about its usefulness and scientific value. What is better about the term "raft" than "biophysics" is that we hope to be able to define the "raft" as we learn more about it in the near future. We will obtain a correct definition of raft when we really understand the membrane domain that is now vaguely called the "raft domain". To this end, we need a working definition for the "raft" and we have to make it useful for investigations of rafts. Therefore, in the context of seeing the membrane as non-ideal liquid mixture of molecules with various levels of miscibilities and also considering that the raft domains are involved in assembling molecules, we propose calling a molecular complex a "raft" when it involves more than two molecules (i.e. three or more molecules), and its formation requires the interactions of cholesterol and a saturated alkyl chain(s). This working definition may be surprising for many researchers,

because it includes very small molecular complexes as rafts. However, it is logical as well as useful, in the sense that it does not preclude anything without specific reasons (two-molecule complexes were excluded from the raft in this definition because one molecule is cholesterol and so the binding of another molecule would not contribute to enhancing molecular interactions or concentrating molecules).

13.6 The Original Raft Hypothesis

The proposal of a raft hypothesis was initially thought to be simple as well as (or because of its simplicity) fascinating (Brown and London 1998; Simons and Ikonen 1997; Simons and Toomre 2000; Simons and van Meer 1988). In the plasma membrane or the Golgi membrane, there may be many micron-sized domains of a liquid-ordered phase, consisting of glycosphingolipids, sphingomyelin and cholesterol, in which specific receptors (GPI-anchored receptors and some selected transmembrane receptors) and cytoplasmic signaling molecules, anchored to the cytoplasmic leaflet of the plasma membrane via saturated alkyl chains (such as Lyn or H-Ras), are concentrated, since these molecules have high affinities for the liquid-ordered phase. The ligand-induced enhanced partitioning of receptor molecules, perhaps due to clustering, into these pre-existing raft domains may trigger and facilitate the downstream signaling. This concentration of specific signaling proteins and the exclusion of other molecules (such as CD45) might play key roles in (de)selecting specific downstream signaling pathways.

13.7 Are there Raft Domains in Steady-state Cells in the Absence of Extracellular Stimulation?

Subsequent research revealed that some modifications may be necessary for the original raft hypothesis.

13.7.1 Standard Immunofluorescence or Immunoelectron Microscopy Failed to Detect Raft-like Domains in the Plasma Membrane of Steady-state Cells

In steady-state cells (in the absence of extracellular stimulation), membrane domains of several hundred nanometers or greater in diameter could not be found using conventional immunolabeling techniques, by either optical or electron microscopy. This suggests that the raft domains are small and the concentration of single species of raft-candidate molecules into a raft domain may not happen. There have been reports suggesting the presence of micron-sized raft domains, but these experiments almost always include the process of crosslinking (often

called chemical fixation) and/or lowering of temperature. Taken together, these results indicate that the micron-sized raft domains were not present in steady-state cells, but were *induced* by the crosslinking of raft-associating molecules or by the cold-enhanced assembly of molecules (including assembly due to exclusion from solidified domains). In chemotactic cells, large raft-like domains have been observed, but these can also be classified into induced rafts, as the cells were already actively engaged in crawling (Manes et al. 2003; Pierini et al. 2003).

The use of low concentrations of paraformaldehyde, which is generally assumed to "fix" the amino-containing molecules at their intrinsic locations, actually enhances the clustering of raftophilic molecules rather than fixing these molecules *in situ*, probably because sphingolipids and cholesterol, which are critically involved in raft formation, cannot be crosslinked by paraformaldehyde (Mayor et al. 1994). For observations at the light microscopy level, the use of at least 3% paraformaldehyde has been recommended (Mayor et al. 1994), whereas for observations at the electron microscopic level, the inclusion of (at least) 0.1% glutaraldehyde in the fixation medium appears to be essential (Hancock 2003; Mayor et al. 1994; Parton and Hancock 2001; Prior et al. 2003). For further details, see Subsection 13.9.1. Any milder chemical fixation may in fact induce enhanced assembly of molecules, leading to signaling events in the plasma membrane. Very often the oligomerization of molecules itself is already the beginning of the signaling events in the plasma membrane.

13.7.2
The Recovery of a Molecule in Detergent-resistant Membrane (DRM) Fractions Might Infer its Raft Association in the Cell Membrane, but the Relationship between DRM Fractions and Raft Domains is Complicated

The partitioning of a molecule in the liquid-ordered domain in artificial model membranes correlates well with its recovery in cold DRM fractions (London and Brown 2000; Schroeder et al. 1998). This inspired the thought that the lipid raft domain in the membrane is the domain in the liquid-ordered phase, and that a high correlation exists between the molecules recovered in the DRM fraction and those partitioned into raft domains in the membrane (reviewed in Simons and Vaz 2004). This has even reached the point in which the DRM association of a molecule has been accepted widely as the biochemical definition of its being a raft-associating molecule. However, there is no direct evidence that a molecule associated with the DRM fraction mostly resides in raft domains in the membrane *in situ*. Rather, cold-detergent treatment might induce macroscopic (of the order of a micron) precipitation of raftophilic molecules (Heerklotz 2002, 2003).

The following is our opinion on how to deal with the relationships between the DRM association and the raft partitioning of a molecule. It will still be correct that the DRM association of a molecule indicates a good possibility that it is associated with the raft domains in the plasma membrane and that the deter-

mination of DRM association is a good starting point for investigating a molecule's raft association in the plasma membrane. However, one has to clearly understand that DRM fractions and rafts are likely to be quite different. It will be perfectly reasonable to treat molecules that show a tendency for DRM association as raft-candidate molecules, but one has to realize that the DRM association of a molecule does not directly imply its raft association in the membrane. Therefore, in the biochemical literature, it is particularly important to make this distinction clear (Mayor and Rao 2004). DRM-associating molecules should not be called "raft" molecules. This loose terminology in the lipid raft literature is causing much confusion in raft-domain research. The DRM-associating molecules could simply be called "DRM molecules", or perhaps "raft-candidate molecules" or "raftophilic molecules" may be acceptable (Kusumi et al. 2004; Subczynski and Kusumi 2003).

In addition, the DRM association has to be described quantitatively, which is rarely done in the literature. For the majority of "DRM" molecules described in the literature, less than half of the total amounts of these molecules were associated with the DRM fractions. Therefore, another important reminder is that the "DRM molecules" described in the literature may actually be associated more frequently with non-DRM than with DRM fractions. To determine the level of DRM association of a molecule in the literature (even semiquantitatively), the information contained in the abstract of the paper is usually insufficient, and one has to look at the actual data with the hope that the gel-patterns presented in figures are representative and reveal the actual amounts of DRM association. There is an urgent need that this situation in raft research be improved.

Furthermore, many biochemical reports conclude that the level of DRM association changed, based on incorrect normalization methods. Often, the total amount of protein in each sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) lane is normalized to be the same. In many cases, this is a correct normalization method. However, in other cases where the changes in the raft association of a molecule are investigated, if such a normalization method is employed, then the changes in the partitioning (between DRM and non-DRM) of the target molecule cannot be evaluated (it shows how the relative amount with regard to all of the other molecules in the lane is changed and so if, for example, much more actin is recovered in DRM fraction after stimulation, then the amount of the molecule of interest in the DRM fraction may appear to be decreased after stimulation, even when the actual amount either did not change or even increased). A correct comparison can be made by loading equal "volumes" (or the total protein) from each fraction after sucrose-gradient centrifugation and keeping all conditions the same before and after stimulation. Regarding "keeping all the conditions the same", it is particularly important to keep both the total number and the density of cells used for the experiments constant because, when cold detergent extraction is carried out, both the concentration of the detergent and the ratio of the cell number/detergent concentration have to remain the same. In the absence of these precautions, reports of changes of DRM association are not useful.

13.7.3
The Size of Rafts in Plasma Membranes of Steady-state Cells may be 10 nm or Less

Sharma et al. (2004), using fluorescence lifetime imaging microscopy based on homo-fluorescence resonance energy transfer (FRET, between two of the same fluorescent molecules) between GPI-anchored proteins, folate receptor or Green Fluorescent Protein (GFP)-GPI, showed that 20–40% of these proteins may be in cholesterol-dependent clusters smaller than pentamers (below 5 nm), with the remaining 60–80% existing as monomers (Sharma et al. 2004; Varma and Mayor 1998). Such low levels of clustering of raftophilic molecules are consistent with the difficulty or variability in detecting hetero-FRET (between two different dye molecules) between raftophilic molecules. Due to the low clustering levels of GPI-anchored proteins or raftophilic molecules, the detection of hetero-FRET appears to strongly depend on the molecules, the cells, the relative concentration of the fluorescent probe molecule among other raftophilic molecules, the size of the region where measurements were made and other subtle variations in the experimental protocols among different laboratories (Feder et al. 1996; Glebov and Nichols 2004; Kenworthy and Edidin 1998; Kenworthy et al. 2000; Nagle 1992; Nichols 2003).

Direct evaluations of the raft size have been carried out in a series of elegant quantitative immunoelectron microscopy studies by Prior, Parton and Hancock (Parton and Hancock 2004; Prior et al. 2003; Plowman et al., personal communication). First, they greatly enhanced the labeling efficiency of their target molecules with their colloidal gold probes (probably over 50%) by employing 4-nm diameter gold particles with careful tuning of the antibody-conjugation method. Second, the images showing the distribution of gold probes bound to various raftophilic molecules (mostly localized on the inner surface of the membrane like H- and K-Ras) were digitized and subjected to a statistical analysis of Ripley's K function to detect the non-random distribution of the gold probes. They initially found 40-nm diameter raft domains that concentrate raftophilic molecules, like H-Ras (before its activation). However, this was further refined recently by considering the geometry of the bound IgG with respect to the gold particles, which gave around 15 nm as the diameter of the steady-state rafts (Parton and Hancock 2004).

Partitioning of the plasma membrane into compartments of 30–200 nm (the size depends on the cell type; Category 2 domain described in Section 13.3 and Fig. 13.2) due to the membrane skeleton fence and the anchored transmembrane protein pickets provides an interesting way to look into the raft size. In contrast to the situation of artificial membranes without partitioning, where the cluster size of the diffusant hardly affects the diffusion rate (Peters and Cherry 1982; Saffman and Delbrück 1975; Vaz et al. 1982), the diffusion rate in the cell membrane is a very sensitive monitor of molecular clustering. Upon clustering, membrane molecules, which rapidly hop across the picket-fences between the compartments as monomers, exhibit dramatically reduced hop rates (oligomeri-

zation-induced trapping, as described in Section 13.3 and Fig. 13.3). If the raft is larger than several nanometers and is stable (its lifetime and the residency time of its constituent molecules are long), one would expect that the diffusion coefficients of the raft-associating molecules would be much smaller than those for the membrane molecules that do not associate with raft domains. The results obtained by Vrljic et al. (2002) and Kenworthy et al. (2004) were at odds with this expectation. The diffusion rates were not different between these types of molecules in the majority of the cases and even when they were different, raft partitioning was not the cause for the difference.

Direct observations of the hop diffusion were carried out by Suzuki et al. using single-molecule techniques (at both 25-μs and 33-ms resolutions, using single-particle tracking and single fluorescent molecule video imaging) (Anderson and Jacobson 2002; Suzuki et al. 2001, 2002, 2003). Both the typical raft-preferring GPI-anchored receptor (GPI-AR) CD59 and the typical non-raftophilic, unsaturated phospholipid DOPE undergo short-term confined diffusion within a compartment of about the same size and long-term hop movement between the compartments, as expected. What was striking was the hop rate. Both molecules exhibited average hop rates that were basically the same, once every 25 ms (in the case of T24 epithelial cells). Taken together with the data by Vrljic et al. (2002) and Kenworthy et al. (2004), these results suggest that the raft size is much smaller than the average gap size between two transmembrane picket proteins, which was proposed to be 2–9 nm (see Section 13.7.4, also see Fig. 2 of Kusumi et al. 2004). Therefore the size of the raft in the steady state may be perhaps of the order of around 2 nm.

However, one has to be careful about this proposed raft size, estimated based on the diffusion measurements. It relates to the size that could fit into the gap between two transmembrane protein pickets anchored to and lined up along the membrane skeleton fence, and thus it is related to the size in the hydrophobic domain. These results suggest that the steady-state raft domains may have a mushroom (for those existing in a single layer of the membrane)- or dumbbell (for those spanning the whole membrane)-type shape with a size of about 10 nm in their hydrophilic part and around 2 nm in their hydrophobic part. This is probably made possible by the flexibility of glycochains that link the protein moiety and the phosphatidylinositol moiety, allowing assembly of saturated chains of GPI and cholesterol somewhere beneath the cluster of the protein moieties (Fig. 13.5, also see below). Furthermore, since the protein moiety is flexibly connected to the hydrophobic core region of the raft, the cluster of the protein moieties is likely to undergo rapid confined thermal oscillative motion on the membrane surface with respect to the hydrophobic raft core (which should undergo much slower diffusion due to 100-fold higher viscosity in the membrane), which would allow for the rapid passage of the cluster of the GPI-AR's protein moiety through the gap between two protruding extracellular hydrophilic domains of transmembrane protein pickets.

In line with these observations, McConnell and his colleagues have advanced the concept of the condensation complex (Anderson and McConnell 2001, 2002;

McConnell and Radhakrishnan 2003; McConnell and Vrljic 2003; Okonogi et al. 2002; Radhakrishnan et al. 2000, 2001), which might consist of 15–30 molecules (Radhakrishnan et al. 2000). The relationship between the condensation complex of cholesterol and saturated lipids with rafts has not been clarified.

Anderson and Jacobson (2002) proposed the model of a "lipid-shell" surrounding raft-associating protein molecules, based on the protein:lipid molar ratio in the DRM fraction (1:80). Eighty molecules of lipids would occupy a membrane area with an overall diameter of 7 nm in a single (outer) leaflet of the bilayer (assuming about a 1:1 molar ratio of polar lipids and cholesterol) wrapped around a raftophilic protein. This model is weak and not well supported by experimental data in several respects. First, there is no direct evidence that the protein:lipid ratio in the raft *in situ* is the same as that in DRM fractions. Second, it is difficult to think about any molecular interaction that could hold 80 molecules of lipids around a GPI-anchored or transmembrane protein. Anderson and Jacobson might have thought of the idea of "boundary lipids" around a transmembrane protein, but the residency time of a lipid in the boundary region is generally limited to about 0.1 μs (East et al. 1985; Horvath et al. 1988; Kusumi et al. 2004), i.e. much too short to have any effect on lateral diffusion

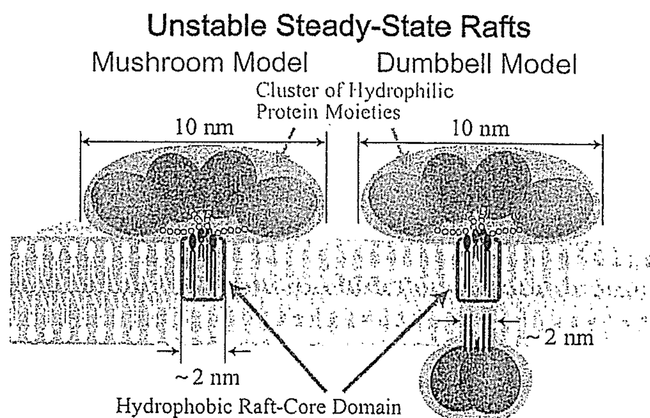

Fig. 13.5 Mushroom and dumbbell models for a small, unstable steady-state raft containing several GPI-anchored receptor molecules. Note that only a fraction of the GPI-ARs may be in such a complex and that the lifetime of such a steady-state raft may be short. The key feature of these models is the flexibility of the GPI-AR glyco-linker, which allows relocation of the PI moieties beneath the complex of hydrophilic protein moieties, leading to complex formation between cholesterol and the saturated alkyl chains of GPI from different molecules in the cluster. The large hydrophilic cluster of the protein moieties may be able to undergo rapid oscillative thermal motions on the membrane surface, due to the low viscosity of the aqueous domain and the flexibility of the glyco-linkers, facilitating rapid passage through the gap between two anchored protein pickets at the compartment boundaries. The protein moiety of this cluster may have a size of about 10 nm in diameter, whereas the hydrophobic core region of such a raft may have a diameter of only about 2 nm, which is much smaller than the average gap size between the pickets (2–9 nm).

of proteins in membranes. Third, although short-lived boundary lipids are detectable by spin-label electron paramagnetic resonance (EPR) spectroscopy, shells of lipids have never been detected by EPR or ^{1}H-nuclear magnetic resonance (NMR) of lipid probes in biological membranes. Fourth, a lipid shell extending to 7 nm diameter in both hydrophobic and hydrophilic domains is much greater than the gap between two picket transmembrane proteins lined up along the membrane skeleton (see Section 13.3), and thus the hop rate for such a large domain across the compartment boundaries in the plasma membrane should be substantially slower than that for single molecules of non-raft phospholipids and proteins, in contrast to actual diffusion measurements of raftophilic molecules and non-raft single molecules. As described above, the average gap between two pickets in a picket line may be about 2–9 nm (see Fig. 2 of Kusumi et al. 2004). This (apparent) contradiction has been explained by Anderson and Jacobson by assuming (1) that the lipid shell would have a longer lifetime than its residency time within a compartment, which is of the order of 1–25 ms, and (2) that the lipid shell is very soft and plastic, and thus may be able to readily cross the picket line. Lipids are unlikely to reside around proteins for as long as several milliseconds and at the same time have a plasticity that has essentially no effect on the hop rate across picket lines.

Single-particle tracking in the early era and photonic force microscopy suggested that the size of the raft ranges between 50 and 200 nm in diameter (Dietrich et al. 2002; Pralle et al. 2000; Sheets et al. 1997). The problem with both of these results is that gold or latex particles have been employed as probes that have tendencies to induce crosslinking. In this regard, much improved gold particles, but not latex beads, have been developed since these early experiments have been performed. In fact, when these improved single-particle probes are used, one no longer or only rarely observes raftophilic molecules confined to transient confinement zones, which are sites where the molecules temporarily stop diffusion (Kusumi and Jacobson, unpublished results). These transient confinements zones have often been interpreted to represent stabilized rafts.

13.7.4 Mushroom Model for the Steady-state Raft

In this section, we will first discuss the size and the shape of GPI-anchored proteins, and then, based on the structure of GPI-anchored proteins, we will reconsider how the proposed raft sizes and the number of molecules involved in a raft may be related. Keep in mind that we are still thinking about *rafts in steady-state cells*. The molecular sizes of GPI-anchored proteins can be roughly evaluated based on X-ray crystallographic data, assuming that the shapes of the protein moieties of these molecules are approximated by rectangular shapes: 16–34 (the expected height direction from the membrane) × 5 × 3 nm for decay-accelerating factor (CD55, the large uncertainty in the height direction is due to the undetermined structure of a part of the protein moiety) (Lukacik et al. 2004), 5 (the expected height direction from the membrane) × 8 × 3 nm for CD59 (Rudd

et al. 1997) and 7 (the expected height direction from the membrane) × 10 × 5 nm for the native dimer of placental alkaline phosphatase (PLAP) or the related shrimp alkaline phosphatase (de Backer et al. 2002; Le Du et al. 2001; Lehto and Sharom 2002). These results suggest that a 10-nm diameter protein portion of a single raft might be able to include only one to four molecules of GPI-anchored proteins, consistent with the evaluation by Sharma et al. (2004).

Consider as an example that is consistent with the data of Sharma et al. (2004), a cholesterol-dependent dimer or a tetramer of GPI-anchored proteins forming a raft domain. (This may be an oversimplification because many more GPI-anchored proteins may exist in the same membrane as monomers that are in equilibrium with these oligomers). Since alkaline phosphatases naturally occur as dimers, they could give a zeroth-order estimate for the distance between the two phosphatidylinositol (PI) molecules beneath the dimer of GPI-anchored proteins. Based on Fig. 8 in Lehto and Sharom (2002), the distance between the two carbonyl termini of the alkaline phosphatase dimer is about 4 nm. This is much greater than the size of the hydrophobic core of the raft, 1–2 nm, expected from the diffusive behavior of CD59 and other raftophilic molecules. Therefore, these results suggest that the flexible glyco-linker of a GPI-anchored protein allows for closer positioning of the two GPI-anchoring chains beneath the GPI-anchored cluster (Fig. 13.5). Within these approximately 2-nm diameter hydrophobic raft-core domains, two to four phospholipid molecules or three to six cholesterol molecules can be accommodated (assuming 0.65 and 0.44 nm^2 for their cross-sections, respectively) (Subczynski and Kusumi 2003). Such an estimate suggests that a steady-state raft with GPI-anchored proteins may contain one to four GPI-anchored proteins, a few molecules of glycolipid/phospholipid and a few cholesterol molecules. This is indeed a very small number of molecules located in a single raft. These molecules probably correspond to the molecules that stay in the hydrophobic core of the raft for prolonged periods of time, i.e. at least the residency time (1–25 ms) of GPI-anchored proteins within a membrane compartment made by plasma membrane partitioning. Since the hydrophilic part of this complex is expected to be on the order of 10 nm in diameter and the hydrophobic core part is thought to be of the order of around 2 nm in diameter, we call this model the "mushroom model" for steady-state rafts present in the outer leaflet of the plasma membrane (Fig. 13.5).

The hydrophobic part of the raft may extend slightly over this core region, but the raftophilic molecules that may be recruited to this extended region, particularly the raftophilic *lipid* molecules (because protein molecules cannot have easy access to the center of the raft, due to steric hindrance in the protein moieties), are likely to have a very short residency time in this domain. Its duration may be much shorter than the residency time of the raft within the membrane compartment. If not, the steady-state rafts could not hop as fast as single molecules of a non-raft phospholipid, DOPE. EPR spin-labeling experiments suggested that the rafts including hemagglutinin (HA) and cholesterol in the influenza virus envelope (plasma) membrane may be short-lived, and/or that the raftophilic probe molecules rapidly diffuse in and out of the raft, on a timescale

of 100 µs or less (Kawasaki et al. 2001), consistent with the short residency time of raftophilic molecules in the extended raft region around the core raft domain.

13.8
Stabilized Rafts Induced by Protein Clustering in Plasma and Golgi Membranes

13.8.1
Clustering of Raft Molecules by Ligand Binding or Crosslinking Induces Stabilized Rafts ("Receptor-cluster Rafts")

In contrast to a cell in the steady state (in the absence of extracellular stimulation), abundant evidence exists for the formation of greater, stabilized rafts upon stimulation by liganding or crosslinking raftophilic molecules, by the recruitment of raftophilic molecules in a cholesterol-dependent manner, to clusters of the activated receptor (Baird et al. 1999; Cherukuri et al. 2004a,b; Dietrich et al. 2001; Dykstra et al. 2003; Field et al. 1995; Harder et al. 1998; Janes et al. 1999; Pierce 2002; Pierini et al. 2003; Pierini and Maxfield 2001; Sheets et al. 1999; Shvartsman et al. 2003; Stoddart et al. 2002; Thomas et al. 1994; Young et al. 2003). These recruited raftophilic signaling molecules might include lipid-anchored signaling (raftophilic) molecules in both the outer and inner leaflet of the plasma membrane, which might facilitate interactions between different signaling molecules in/on a single receptor-cluster raft, leading to the activation of a signaling pathway.

Upon *de novo* synthesis of certain raftophilic molecules, they may be clustered in the Golgi (Paladino et al. 2004), perhaps by certain molecules that recognize them there. Such molecules have not been identified, but those that recognize GPI-anchored proteins may be strong candidates for such crosslinking/recognition molecules. The formation of clusters of GPI-anchored molecules would induce stabilized rafts in the Golgi membrane, possibly leading to enhanced trafficking to specific membrane compartments in the cell (Paladino et al. 2004).

13.8.2
How can Raft Molecule Clustering Induce Stabilized Rafts?

How can the clustering of GPI-anchored proteins (induced by ligands, lectins, pathogens or other reagents) induce greater, stabilized rafts? What makes the clustered raft molecules different from the monomeric raft molecules, i.e. what is the mechanism for recruiting the downstream signaling molecules only to the receptor-cluster rafts and not to steady-state rafts (see, e.g. Field et al. 1997; Pribluda et al. 1994; Sheets et al. 1999; Wu et al. 2004, for the case of the Fcε receptor)?

As an example, consider the hypothetical oligomerization of eight GPI-AR molecules, which induces the close assembly of eight to 16 saturated alkyl

chains. Note that since the protein moiety of a GPI-AR molecule is much larger (say 5 nm in diameter) than the two alkyl-chain anchors (say 0.6×1.2 nm), there is always enough space for other lipids to stay around the (8 to 16) anchoring chains in a GPI-AR cluster (say 15 nm in diameter). In such a GPI-AR cluster, eight to 16 saturated alkyl chains are concentrated and confined beneath the cluster of hydrophilic protein moieties of these GPI-AR molecules, and they may be brought close to each other because the glycochain that links the PI with the protein moiety in a GPI-AR is probably flexible (Lehto and Sharom 2002). We suggest that this concentration of saturated chains of GPI-ARs and their freedom of relocation within the GPI-AR cluster would be responsible for the formation, in the hydrophobic domain in the membrane, of (transient) complexes of these GPI-ARs' saturated alkyl chains together with cholesterol, glycosphingolipids, sphingomyelin and the saturated-lipid-anchoring chains of other lipid-anchored proteins, which would lead to the formation of enlarged, stabilized receptor-cluster rafts.

How might the concentration (plus confinement) and the relocation capability of the GPI-ARs' saturated anchoring chains beneath the GPI-AR cluster induce the recruitment of cholesterol and other raft components for the formation of receptor-cluster rafts?

First, cholesterol mixes well with saturated chains, while it tends to be excluded from the bulk, disordered liquid domains because of its poorer miscibility with unsaturated alkyl chains (Pasenkiewicz-Gierula et al. 1991; Subczynski et al. 1990). Therefore, if given a choice, cholesterol would move to the region where the saturated chains are concentrated.

Second, the long alkyl chains of sphingolipids might also be excluded from the bulk, disordered-liquid domain, due to mismatches in their hydrophobic lengths, particularly when the long saturated chains are in contact with cholesterol (Bretscher and Munro 1993; Gil et al. 1998; Kusumi and Hyde 1982). Thus, they tend to assemble in the clusters of GPI-AR–cholesterol, where the hydrophobic chains are longer and the membrane is thicker than in the bulk domain of the membrane.

Third, importantly, since the saturated alkyl chains of GPI-AR are concentrated and confined in the GPI-AR cluster, and can relocate within the cluster, the complex formation with cholesterol and other molecules with saturated alkyl chains beneath the cluster of the protein moieties of GPI-ARs may be greatly facilitated.

Fourth, since the cholesterol-saturated alkyl chain interaction enhances the *trans* conformation (Pasenkiewicz-Gierula et al. 1991; Subczynski et al. 1990), and thus orders saturated alkyl chains in the GPI-AR region, the thermal mobility in this region is reduced and the lifetime of the receptor-cluster rafts as well as the residency time of cholesterol, glycosphingolipids and sphingomyelin in this region is increased.

However, it is important to realize that the constituent lipid molecules of such "receptor-cluster rafts" may still be able to exchange with those in the bulk disordered-liquid domain, just like the lipids moving back and forth between the

ordered- and disordered-liquid-phase domains in artificial membranes (Simons and Ikonen 1997).

The receptor-cluster rafts are rafts that are directly involved in biological functions. The considerations given in this section suggest that the receptor-cluster rafts are poised to form in resting-state cells and that only small modulations of the delicate balance of molecular interactions, such as the clustering of GPI-ARs, are required to form receptor-cluster rafts. Therefore, the small, unstable steady-state rafts that are present before stimulation can be easily transformed into larger receptor-stabilized rafts.

13.9
Can Receptor-cluster Rafts Work as Platforms to Facilitate the Assembly of Raftophilic Molecules?

One of the keys for understanding the mechanism of raft-mediated or raft-facilitated signaling events may be how the raft may help to recruit and concentrate signaling molecules (Janes et al. 1999; Tavano et al. 2004). As a prelude to solving this issue, many studies have been performed to examine whether the crosslinking of a raftophilic molecule may induce the colocalization of another raftophilic molecule (with or without crosslinking). In the first subsection, we summarize the technical problems related to such immuno-colocalization experiments. In the following subsection, the results of colocalization experiments, mostly using immunofluorescence methods, will be presented (with supportive data using pull-down and immunoprecipitation assays). In the last subsection, we summarize the lack of robustness in such colocalization experiments, with variations and low levels of quantitative reproducibilities among different raftophilic molecules and cells, and subtle variations in the protocols from different laboratories.

13.9.1
Benchmarks for Experiments Examining the Colocalization of Raftophilic Molecules

Mayor et al. (1994) observed the colocalization of GPI-anchored proteins, folate receptor, CD55 and Thy-1, under various conditions using immunofluorescence microscopy. The experimental procedures and analysis described in this report in many respects represent the benchmark for such studies, and one should follow these protocols when this type of colocalization study is intended.

1. The normal fixation protocol using paraformaldehyde, employed in many studies, tends to induce the clustering of GPI-anchored proteins, rather than blocking it. To block the redistribution of GPI-anchored proteins by chemical fixation, the fixation with 3% paraformaldehyde has to be performed for over 1 h or with the inclusion of 0.3–0.5% glutaraldehyde (20–30 min). These fixation conditions have to be tested out for individual experimental systems.

2. Fixation should be carried out at room temperature without further lowering of the temperature, to avoid cold-enhanced formation of particular membrane domains. From today's viewpoint, it would be wise to compare the results with those obtained with fixation at 37 °C.

3. The colocalization levels of these GPI-anchored proteins have not usually been very high (even when colocalization appears to occur significantly by eye). They have been in the range of 35–55% in the case reported by Mayor et al. (1994). Since the colocalization levels are relatively low in this type of experiment, it is important to realize that the evaluation of colocalization must be done quantitatively and the random colocalization value must be given, as Mayor et al. did (also see Parmryd et al. 2003). Such random colocalization values can be conveniently obtained by shifting two superimposed images by, say, 1 μm relative to one another (Koyama-Honda et al. 2005).

4. Mayor et al. (1994) clearly described the colocalization percentages of one molecule's spots with the other molecule's spots. In many published studies, the relationships between molecules were not quantitatively described and, therefore, these results are less meaningful for obtaining quantitative measures of colocalization.

5. When membrane molecules are clustered, they tend to become colocalized in clathrin-coated pits and caveolae. If such events take place, then the colocalization of two molecules may not necessarily mean mutual binding to each other and may simply suggest recruitment to the same site. Therefore, when clustering is induced, colocalization with clathrin-coated pits, caveolae and the other internalization apparatus on the cell surface has to be examined, as done by Mayor et al. (1994).

6. In addition, other normal controls, like the partial depletion of cholesterol and the observation of non-raft molecules, such as transferrin receptor and unsaturated phospholipid, should be performed. The second control is important to do in addition to the cholesterol depletion control, as the antibody-induced clustering of raftophilic molecules may induce undulation and accumulation of the membrane in/near the clustered domain (Glebov and Nichols 2004).

13.9.2 Simultaneous Crosslinking of Two GPI-anchored Receptors

Mayor et al. (1994) found that when folate receptor, CD55 and Thy-1 were simultaneously (a combination of two molecules for each experiment) crosslinked with antibodies, they became colocalized at levels 3–5 times greater than the random colocalization controls. It was likely that the colocalization of these molecules took place in caveolae. Harder et al. (1998) also crosslinked PLAP, Thy-1 or influenza virus HA and the raft ganglioside GM_1 using antibodies and/or cholera toxin. The patches of these raft markers overlapped extensively at 12 °C

and less extensively at 37 °C, but not on the patches of non-raft markers, such as the transferrin and low-density lipoprotein receptors. In this study, it was not clear if the co-patched spots occurred in clathrin-coated pits. However, caveolae were not involved because colocalization also took place in caveolin-free T lymphocytes.

13.9.3
Sequential Crosslinking of One Species of GPI-anchored Receptors Followed by Crosslinking of a Second Species without Fixation

One key issue with the crosslinking of a second molecule appears to be the timing of the second crosslinking, i.e. whether the crosslinking of the second molecule occurs simultaneously with the first one or sequentially after the crosslinking of the first molecule is complete. (No chemical fixation is performed between the two crosslinking steps.)

Few sequential crosslinking experiments have been performed. Mayor et al. (1994), using folate receptor and Thy-1 (the order was permuted) found co-clustering of these molecules after crosslinking of the second species. Perhaps most of this co-clustering occurred in caveolae (see their Fig. 3).

13.9.4
Examination of the Recruitment of Non-crosslinked Second Raftophilic Molecules to Crosslinked GPI-anchored Receptor Clusters

Colocalization experiments were also conducted under conditions where the second crosslinking was omitted. Thus far, all of the observations in this class of experiments were carried out using fixed cells and in most experiments the fixation occurred after the crosslinking of the first GPI-AR. Different groups have obtained different results in different systems as described in the following. Mayor et al. (1994) did not detect any colocalization of the second species (folate receptor or Thy-1) to the crosslinked first molecular species (Thy-1 or folate receptor, respectively). Fra et al. (1994) also found that non-crosslinked Thy-1 or GM_1 was not recruited to pre-crosslinked GM_1 or Thy-1, respectively, in caveolin-free T cell hybridoma 2B2318 cells. Meanwhile, Harder et al. (1998) followed the distribution of non-crosslinked PLAP with patched HA or non-crosslinked HA with patched PLAP. They occasionally observed co-clustering of the two, but the co-patching behavior was quite variable.

Marwali et al. (2003) observed the colocalization of GM_1 with pre-crosslinked Thy-1 (see their Fig. 7). Caution is advised when interpreting these data because the cells were fixed only after the second species was stained. In their protocol, staining of the second species, GM_1, is expected to strongly affect the final results. The distribution of GM_1 was observed with the pentavalent B-subunit fragment of cholera toxin, which thus may collect five GM_1 molecules, and the staining was performed at 4 °C, which might enhance the formation of raft-like domains. Therefore, it is possible that the apparent colocalization of "non-cross-

linked GM_1" may be due to the recruitment of clustered GM_1. A reverse experiment was performed by Mitchell et al. (2002), who found that crosslinked GM_1 recruited the non-crosslinked GPI-AR CD59 (see their Fig. 5).

Interestingly, Harder et al. (1998) found that after crosslinking of PLAP, which is located in the *outer leaflet* of the plasma membrane, the SFK Fyn, which is anchored in the *cytoplasmic leaflet* of the plasma membrane via two saturated alkyl (one myristoyl and one palmitoyl) chains. These two alkyl chains may promote the partitioning of Fyn into raft domains with concentrated saturated alkyl chains beneath the PLAP clusters. This result is very interesting because the cytoplasmic leaflet does not contain appreciable amounts of sphingomyelin and it is not clear how the presence of an ordered lipid domain in the outer leaflet can induce an ordered lipid domain in the cytoplasmic leaflet.

13.9.5
Difficulty in Colocalization Experiments using Raftophilic Molecules: Low Levels of Colocalization and Quantitative Reproducibility Due to Sensitivity to Subtle Differences in Experimental Conditions and Protocols

As suggested in the previous subsections, colocalization experiments using raftophilic molecules turned out to be much more difficult than expected. In many colocalization experiments, the results were so clear that a statistical analysis of colocalization was practically unnecessary. However, in colocalization studies of raft molecules, as described in Section 13.9.1, the level of colocalization is typically only 3- to 5-fold greater than in random controls (Mayor et al. 1994; Parmryd et al. 2003).

Unfortunately, many investigators in this field experienced difficulties in reproducing data obtained by other laboratories. However, the differences tend to be quantitative rather than contradictory in most cases (e.g. 50% colocalization in one lab and 30% in another lab). Often, the robustness of the results appears to be lacking. The results appear to be sensitive to subtle differences in experimental conditions and protocols, and the types of molecules and cells that were used. We believe that this difficulty may be deeply rooted in (1) the essential nature of the colocalization of two raftophilic molecules, one of which may have been pre-clustered, and (2) the chemical fixation used for the observation of colocalization.

13.10
Timescales Again! Transient Colocalization of Raftophilic Molecules

An idea to explain the difficulty in colocalization experiments can be found in the experiments carried out by Shvartsman et al. (2003). These authors tried to observe the interaction between two antigenically distinct influenza HA proteins: a wild-type transmembrane HA and a GPI-anchored HA. They already knew that biochemical and immunofluorescence methods did not reveal any as-

sociation between these two molecular species. They developed a very smart approach for detecting the interaction of crosslinked with non-crosslinked molecular species. In live cells that coexpressed these two proteins, they measured by fluorescence recovery after photobleaching (FRAP) the lateral diffusion rate of wild-type HA before and after the aggregation of GPI-HA into immobile patches. They found that the presence of GPI-HA patches reduced the diffusion rate of wild-type HA, suggesting a *transient* binding of individual wild-type HA molecules to crosslinked GPI-HA.

This result also suggests the possibility that a variety of short-term interactions between raftophilic molecules exist that may be missed in immunofluorescence colocalization experiments and biochemical pull-down assays. Such dynamic on–off interactions or rapid association–dissociation may lie at the edge of the detectability limit of static immuno-colocalization observations, which involve the chemical fixation of transiently colocalized molecules. Such dynamic effects make the detectability of colocalization even more sensitive to subtle variations of molecules, cells and experimental protocols or reagents, leading to low levels (and thus large fluctuations) of static colocalization and poor quantitative reproducibility of the data from different laboratories or sometimes even among different researchers in the same group.

The results described in Sections 13.9.2 and 13.9.4 can be summarized as follows.

1. When two species of raftophilic molecules are individually crosslinked, they tend to show colocalization (although the quantitative level of colocalization may vary under different conditions).
2. When one species of raftophilic molecule is crosslinked and then the recruitment of another molecular species of raftophilic molecules is observed, the results are variable (even when the experiments are done by the same researchers; see, e.g. Harder et al. 1998) and colocalization is more difficult to detect.

We propose an explanation for these results by considering the dynamics of the interacting molecular species, i.e. *the timescales of interaction* (Fig. 13.6). We think that the interaction is transient, especially when the second molecular species is not crosslinked (Fig. 13.6, left), but also when two clusters are separately crosslinked (Fig. 13.6, right). If one chemically fixes such dynamical membrane systems, the level of colocalization will depend on both the frequency and duration of the molecular interactions, making colocalization experiments very sensitive to experimental details. This is true for the case where both of the two molecular species are crosslinked (Fig. 13.6, right) and even more so when the second molecular species is not crosslinked (Fig. 13.6, left). Single-molecule approaches may be the key to resolving the dynamics of such transient colocalization interactions. Efforts to directly observe homo- and heterotypic colocalization events at the single molecule level are underway in our laboratory.

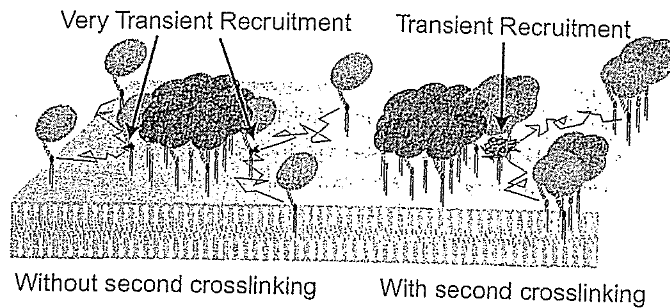

Fig. 13.6 Model of dynamic recruitment of raftophilic molecules, with or without crosslinking, to receptor-cluster rafts, explaining the lack of robustness in immunofluorescence colocalization experiments using chemically fixed cells. Colocalization events may occur very dynamically – to a stabilized raft of crosslinked GPI-anchored proteins a different species of raftophilic molecules may be recruited transiently. If these dynamic colocalization events are visualized by immunofluorescence after chemical fixation, the observed colocalization level may be low and near the detectability limit, making the colocalization detection sensitive to small experimental variations. Detectability of colocalization depends on the efficiency of chemical crosslinking, temperature, and how exactly crosslinking and chemical fixation are performed. When both of the observed molecular species are clustered, colocalization is observed at much higher rates than when only one of the two species is clustered. This suggests the following dynamic recruitment model: the efficiency of chemical fixation of the second molecule at the stabilized raft of the first clustered molecule becomes much higher after crosslinking of the second molecule because the duration of colocalization will be prolonged after clustering of the second molecule.

13.11 Modified Raft Hypothesis

We think that the original raft hypothesis, described in Section 13.6, is essentially correct, but we propose three modifications to the original model.

1. In the absence of extracellular stimulation or crosslinking of raftophilic molecules, the rafts in these steady-state cells are small and some of them may contain as few as one to four GPI-anchored proteins, together with a few molecules of glycolipids and/or phospholipids with saturated alkyl chains and a few molecules of cholesterol. The cluster size of the hydrophilic protein moieties of GPI-anchored receptors may be of the order of 10 nm in diameter, whereas the hydrophobic core of the steady-state raft may be only around 2 nm in diameter. This is described by the mushroom model (Fig. 13.5). One of the most important characteristics of such a steady-state raft is that the glyco-linker of GPI-AR is flexible, allowing fast oscillative thermal motion of the protein moiety and positional freedom for the GPI moiety beneath the protein moiety. For example, when a four-molecule GPI-AR cluster is formed, the GPI-anchoring chains can be close to each other beneath the cluster of protein moieties.

2. Enlarged stabilized rafts are induced by liganding or crosslinking of GPI-ARs, which leads to GPI-AR cluster formation. The cluster of the GPI-linked protein moieties will concentrate the saturated alkyl chains of the GPI anchors beneath the cluster, which in turn will attract cholesterol molecules, leading to further assembly of glycosphingolipids and sphingomyelin with saturated alkyl chains, and the formation of a receptor-cluster raft.

3. This enlarged stabilized raft forms a platform to facilitate the recruitment and assembly of various raftophilic molecules; however, this recruitment and the associated interactions are likely to occur only transiently.

These ideas and models need further testing. Importantly, all of these events have to be described in a quantitative fashion. Some of the most important quantitations that await experimental verification concern the sizes and lifetimes of steady-state and receptor-cluster rafts, and the duration of colocalization of individual raft components.

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